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The innervation of the pyloric region of the crab, *Cancer borealis*: Homologous muscles in decapod species are differently innervated

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Summary. The muscles of the pyloric region of the stomach of the crab, *Cancer borealis*, are innervated by motorneurons found in the stomatogastric ganglion (STG). Electrophysiological recording and stimulating techniques were used to study the detailed pattern of innervation of the pyloric region muscles. Although there are two Pyloric Dilator (PD) motorneurons in lobsters, previous work reported four PD motorneurons in the crab STG (Dando et al. 1974; Hermann 1979a, b). We now find that only two of the crab PD neurons innervate muscles homologous to those innervated by the PD neurons in the lobster, *Panulirus interruptus*. The remaining two PD neurons innervate muscles that are innervated by pyloric (PY) neurons in *P. interruptus*. The innervation patterns of the Lateral Pyloric (LP), Ventricular Dilator (VD), Inferior Cardiac (IC), and PY neurons were also determined and compared with those previously reported in lobsters. Responses of the muscles of the pyloric region to the neurotransmitters, acetylcholine (ACh) and glutamate, were determined by application of exogenous cholinergic agonists and glutamate. The effect of the cholinergic antagonist, curare, on the amplitude of the excitatory junctional potentials (EJPs) evoked by stimulation of the pyloric motor nerves was measured. These experiments suggest that the differences in innervation pattern of the pyloric muscles seen in crab and lobsters are also associated with a change in the neurotransmitter active on these muscles. Possible implications of these findings for phylogenetic relations of decapod crustaceans and for the evolution of neural circuits are discussed.

Introduction

The pyloric system of the stomatogastric ganglion (STG) of decapod crustaceans is an extremely useful preparation for the analysis both of the neural basis underlying rhythmic movements (Selverston et al. 1976; Selverston and Miller 1980; Miller and Selverston 1982a, b; Eisen and Marder 1982), and of the mechanisms by which these rhythmic movements are modulated or influenced by other neural inputs (Marder and Eisen 1984b; Eisen and Marder 1984; Hooper and Marder 1984; Beltz et al. 1984; Nagy and Dickinson 1983; Dickinson and Nagy 1983; Robertson and Moulins 1981).

The pyloric region of the decapod crustacean stomach is a complex cylindrical pumping and filtering device that food passes through between the gastric mill and the hindgut (Maynard and Dando 1974). An oversimplified description of the function of the muscles of the pyloric region is that they can be divided into dilators and constrictors. In general, the dilators are extrinsic muscles, with one insertion on the surface of the pyloric region of the stomach and the other on the hyperdermis. Contraction of these muscles tends to open the pyloric valve. In contrast, the constrictors are in-

Abbreviations: *ACh* acetylcholine; *Carb* carbamylcholine; *cpv* muscles of the cardio-pyloric valve; *cpv7n* nerve innervating muscle cpv7; *cv* muscles of the ventral cardiac ossicles; *cv1n* nerve innervating muscle cvl; *cv2n* nerve innervating muscle cv2; *EJP* excitatory junctional potential; *IC* inferior cardiac neuron; *IV* inferior ventricular neuron; *IVN* inferior ventricular nerve; *LP* lateral pyloric neuron; *LPG* lateral posterior gastric neuron; *lvn* lateral ventricular nerve; *mvn* medial ventricular nerve; *p* muscles of the pylorus; *PD* pyloric dilator neuron; PD_{in} intrinsic PD neuron; PD_{ex} extrinsic PD neuron; *pdn* pyloric dilator nerve; *PY* pyloric neuron; *pyn* pyloric nerve; *STG* stomatogastric ganglion; *VD* ventricular dilator neuron

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trinsic muscles, and attach to two ossicles on the surface of the pyloric region. Contraction of these muscles tends to close the pyloric valve. However, in reality the muscles of the pyloric region form a complex structure, the operational mechanics of which is not well understood but is a subject of study (LaMon and Miller 1985).

The STG contains about 30 neurons, of which approximately one-half participate in the pyloric rhythm. Most of these pyloric circuit neurons are motor neurons that innervate more than 20 pairs of striated muscles of the pylorus. As a result of activity in the pyloric motorneurons, these muscles move in a rhythmic sequence with a frequency of 0.5–3 Hz. In *Panulirus interruptus*, the spiny lobster, the synaptic connectivity among the pyloric neurons (Eisen and Marder 1982), membrane properties of the pyloric neurons (Russell and Hartline 1978, 1982) and mechanisms responsible for the production of the pyloric motor patterns (Miller and Selverston 1982a, b) have been described. Other decapod species such as *Homarus americanus*, *Homarus gammarus*, *Jasus lalandii*, *Cancer borealis*, *Cancer irroratus*, and *Cancer pagurus* produce pyloric motor patterns that are similar to those produced by *P. interruptus* (Beltz et al. 1984; Hooper and Marder 1984; Robertson and Moulins 1981; Selverston and Moulins 1985). Additionally, the anatomy of the pyloric region is sufficiently similar in different species so that homologous muscles have been defined (Maynard and Dando 1974; Govind et al. 1975; Meiss and Norman 1977b). However, differences in the pyloric neural circuit in different species must exist, since crabs contain four pyloric dilator (PD) motor neurons (Dando et al. 1974; Hermann and Dando 1977; Hermann 1979a, b), instead of the two PD neurons found in lobsters.

The possibility that the pyloric circuit in crabs might be different from that in *P. interruptus* prompted us to investigate the organization of the pyloric circuit in the crab, *Cancer borealis*. As a first step in elucidating the details of the synaptic connectivity among the pyloric neurons, it was necessary to identify properly each class of neurons in the pyloric circuit. In this paper we have studied the innervation of the pyloric region of the stomach of *C. borealis* by the motorneurons of the STG. We recorded intracellularly from the pyloric region muscles during rhythmic pyloric activity, and have defined the times in the pyloric rhythm when each muscle is active. Surprisingly, some of the muscles that in *P. interruptus* are innervated by PY neurons contract during the PD neuron phase of the pyloric rhythm and are innervated by two of the four electrically coupled PD neurons. These differences in innervation pattern between the two species are associated with differences in neurotransmitter responses shown by homologous muscles in the two species. A preliminary report of these data has appeared in abstract form (O'Neil et al. 1985).

Materials and methods

Animals. Cancer borealis were purchased from local (Boston, MA) fishermen, and kept in Instant Ocean sea-water aquaria at 10–12 °C until used. 106 male and female animals (100–500 g) were used.

Physiological saline. C. borealis saline had the following composition (mM/l): NaCl, 440; $MgCl_2$, 26; $CaCl_2$, 13; Tris base, 11; maleic acid, 5.2; pH 7.5–7.6. In some experiments glucose (10 mM) was added to the saline directly before use. All experiments in this paper were carried out with chilled saline (10–13 °C) continuously superfusing the preparation at 4–20 ml/min.

Methylene blue stains. Preparations were stained by a modification of a technique developed by R. Hoy (personal communication). Individual muscles or regions of the stomach were carefully cleared of overlying connective tissue and fat, taking care to preserve the innervation intact. The saline covering the preparation was removed, and several drops of a concentrated (dark blue) solution of methylene blue dissolved in distilled water was placed on the tissue. Small pieces of kimwipe were placed over the region to be stained, and then several drops of methylene blue were again applied. The preparation was placed in the refrigerator overnight. The next day the kimwipes were removed, and the preparation was viewed. This technique provides extremely vivid and reproducible stains. Some preparations were fixed in a saturated solution of ammonium molybdate for 2–3 h, rinsed, dehydrated, and cleared (F. Nagy, personal communication).

Equipment. All electrophysiological equipment was conventional. Data were taken either on 4 channel Gould chart recorders directly, or else taped on a FM tape recorder (Vetter Instruments), and played back onto a chart recorder after an 8-fold reduction in speed.

Electrodes. Intracellular recordings from muscles were done with glass microelectrodes (10–15 MΩ) filled with 2.5 *M* KCl. Intracellular recordings from STG neurons were done with glass microelectrodes of 20–60 MΩ filled with KCl. Iontophoretic electrodes were filled with 1 *M* acetylcholine chloride (ACh) or 1 *M* Na glutamate, pH 8.0. Extracellular recordings and stimulations of nerves were done either with suction electrodes or stainless steel pin electrodes insulated with vaseline.

Attached ganglion and pyloric muscle preparation. The stomach was removed from the animal, split open on the ventral midline, and laid flat in a Sylgard-lined dish. The nerves in the anterior end of the preparation were dissected free of the stomach, and the STG desheathed. This preparation allowed simultaneous intracellular recordings from neuron somata in the STG, extracellular recordings from the motor nerves, and intracellular recordings from the muscles (Fig. 1).

Individual muscle preparations. Muscles were dissected with both origin and insertion attached while taking care to retain

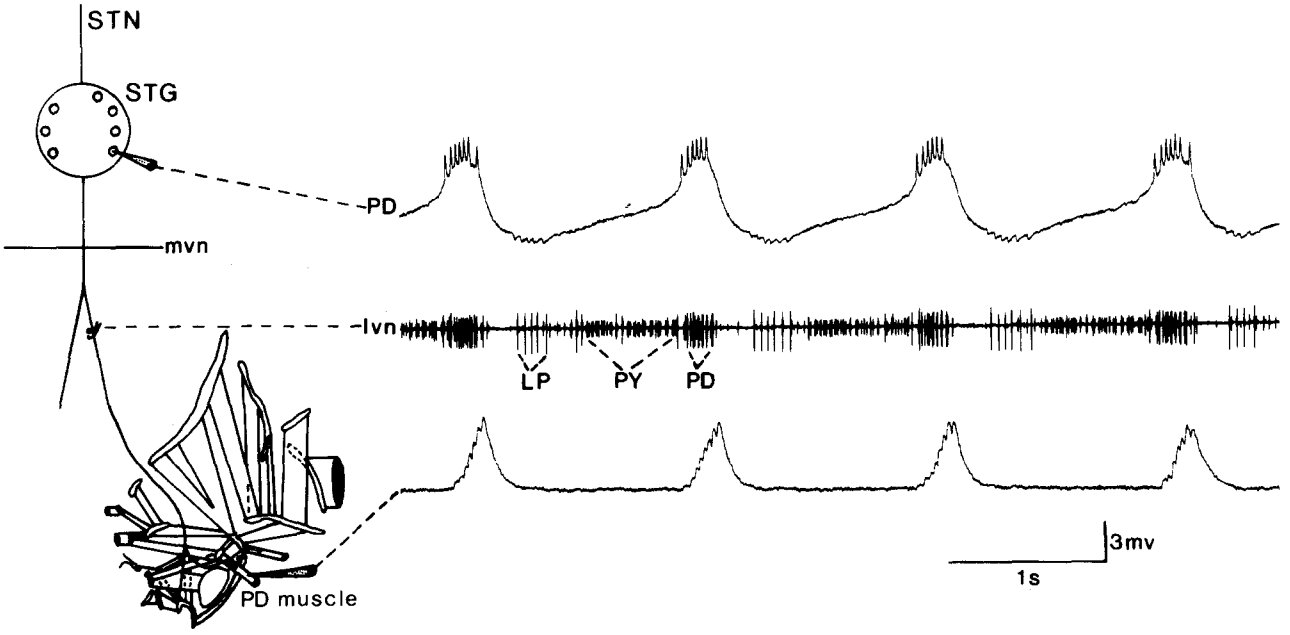


Fig. 1. Pyloric rhythm. Left: schematic representation of the stomatogastric neuromuscular preparations showing the points at which the recordings (right) were made. Right: simultaneous recordings from a PD neuron (top trace: intracellular recording), the lvn (middle trace: extracellular recording) and the cpv2b muscle (bottom intracellular recording)

the innervation intact. A suction electrode was used to stimulate the motor nerve (0.5 ms) at 0.1 to 10 V to evoke EJPs. The voltage was varied to determine the number of motorneurons innervating each muscle.

Pharmacology. All changes in saline composition were achieved without interrupting saline flow by means of a switching manifold. All chemicals were standard, and all drugs were purchased from Sigma Chemical Co. Drug-containing solutions were made fresh directly before use. All experiments involving the application of exogenous agonists were carried out in saline containing 20 m*M* Mn^{++} to block all synaptic transmission (Marder and Eisen 1984a). In experiments using application of agonists (Carb and glutamate) muscle fiber input impedance was monitored by placing two electrodes in a muscle fiber, and passing current into the fiber with one electrode while recording voltage deflections with the second. In these experiments high concentrations of agonist were applied for a short time in the bath (the inflow was switched to wash as soon as the response started to peak). This paradigm avoided problems of desensitization associated with long applications of agonist in the bath, and was more reliable as a test of sensitivity to neurotransmitters than iontophoretic applications. This is because iontophoretic applications often fail to show a response to test agonists if the pipette is placed in the wrong position even if receptors are present on the muscle membrane. However, for most muscles tested, the results were verified using iontophoretic applications of agonists.

Results

The anatomy of the pyloric region was described for several decapod species, including the crab, *Callinectes sapidus*, and the lobster, *P. interruptus* by Maynard and Dando (1974). The anatomy of *C. borealis* is sufficiently similar to that of *C. sapidus* and *P. interruptus* so that homologous muscles in the three species can be recognized, although some differences among the species do exist. To produce a scale drawing of the pyloric region of *C. borealis*, we photographed it through the dissecting microscope, and then traced the position of the ossicles and the muscles (Fig. 2). The nomenclature of Maynard and Dando (1974) is used.

Identification of the pyloric neurons

In principle, the motorneurons in the STG are identified according to which muscle they innervate (Selverston et al. 1976; Mulloney and Selverston 1974). For example, in *P. interruptus* the PD neurons were originally identified as those neurons innervating the dorsal dilator muscles, cpv 1a, b and the pyloric dilator muscles, cpv 2b (Maynard and Dando 1974). In practice, neurons are routinely identified according to which motor nerve their axons take (Selverston et al. 1976). This means in *P. interruptus* the PD neurons are identified as those neurons with axons in the pyloric dilator nerve (pdn), the nerve innervating the pyloric dilator muscles. However, this latter method did not allow us to identify unambiguously several of the motorneurons in *C. borealis*. Therefore we determined in *C. borealis* which neurons innervate each of the pyloric region muscles.

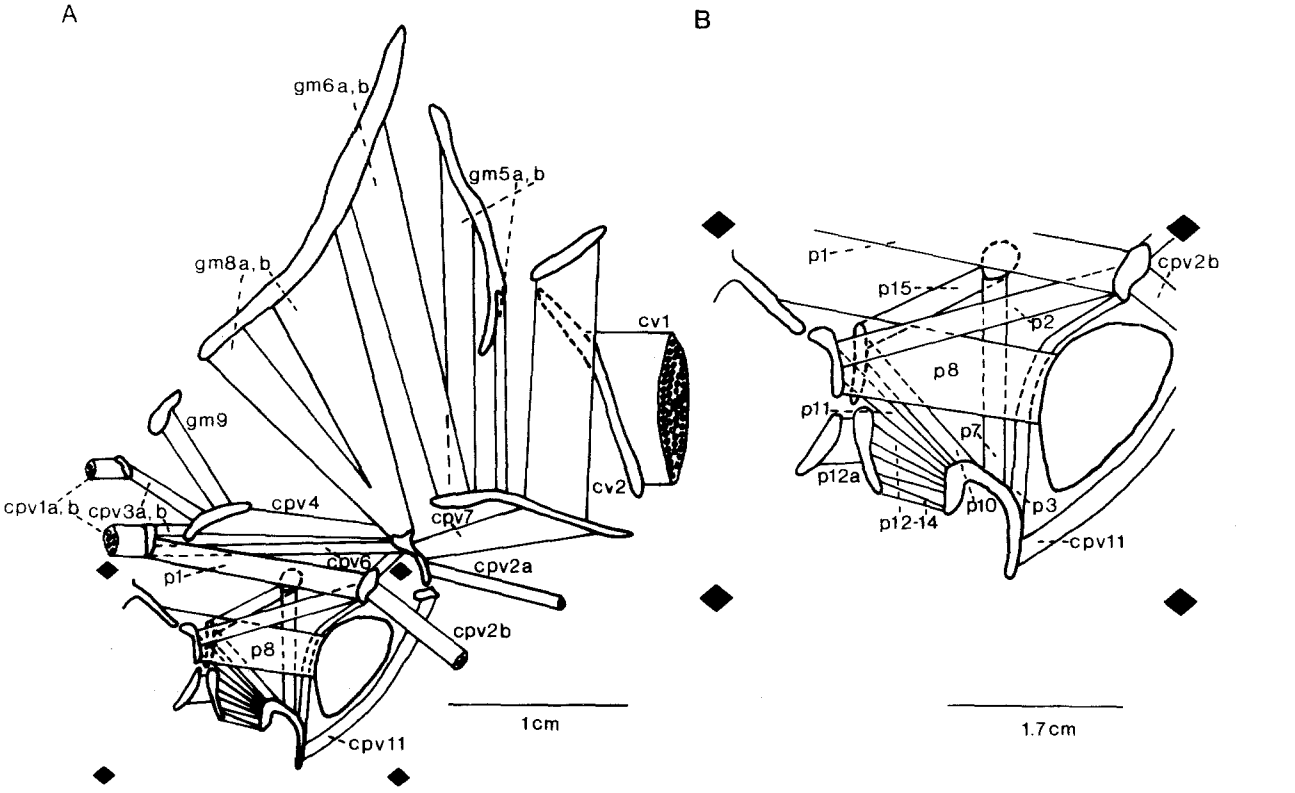


Fig. 2A, B. Pyloric region of *C. borealis*. **A** Scale drawing of the right half of the bilaterally symmetrical pyloric region. In the animal the pylorus is cylindrical with its long axis in the anterior-posterior dimension. A ventral midline cut was made between the two cpv2 muscles, and the stomach laid flat. Thus muscles cpv1a, b are situated at the dorsal midline in the animal, while cpv2a, b are situated at the ventral midline. **B** An enlargement of the region marked with diamonds in **A** (note the scale change)

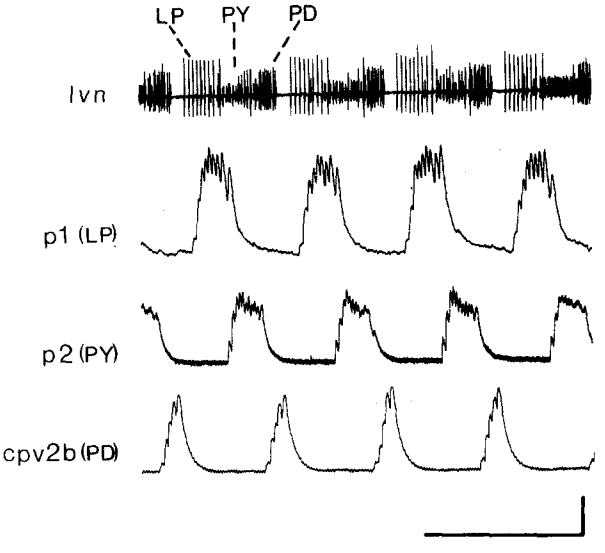


Fig. 3. Activity of pyloric motorneurons. Top trace is an extracellular recording from the lvn. Bottom traces: intracellular recordings from p1, p2, and cpv2b muscles. Resting potentials of the fibers were: p1, −58 mV; p2, −40 mV; cpv2b, −33 mV. Horizontal calibration bar 1 s; vertical bar 2 mV

Figure 3 shows the normal sequence of muscle activity of the crab pyloric rhythm. The top trace is an extracellular recording from the lateral ventricular nerve (lvn), in which bursts from three classes of pyloric neurons can be distinguished. The activity of the Lateral Pyloric (LP) neuron is seen as the largest unit firing. LP neuron activity is followed by PY neuron activity (smallest units) and then by PD neuron firing (middle-sized units). The bottom three traces are intracellular recordings from three pyloric muscles, p1 (LP neuron innervated), p2 (PY neuron innervated) and cpv2a (PD neuron innervated). It is apparent that the normal sequence of pyloric activity consists of a rhythmic alternation in the time of depolarization of the muscles innervated by the LP, PY, and PD neurons. These recordings are similar to those we have obtained from homologous muscles in *P. interruptus*. However, when we looked more closely in *C. borealis* at the innervation of the pyloric region, we also found some striking differences between the two species.

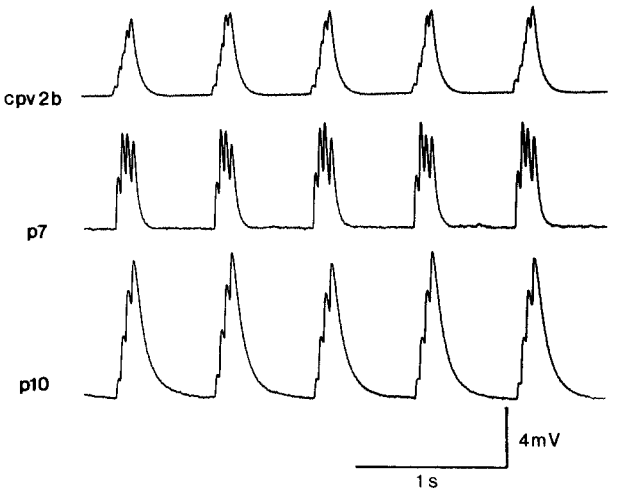


Fig. 4. Simultaneous intracellular recordings from muscles active in the PD-phase of the pyloric rhythm. Muscle cpv2b is an extrinsic muscle; muscles p7 and p10 are intrinsic muscles. Resting potentials: cpv2b, −39 mV; p7, −74 mV; p10, −64 mV

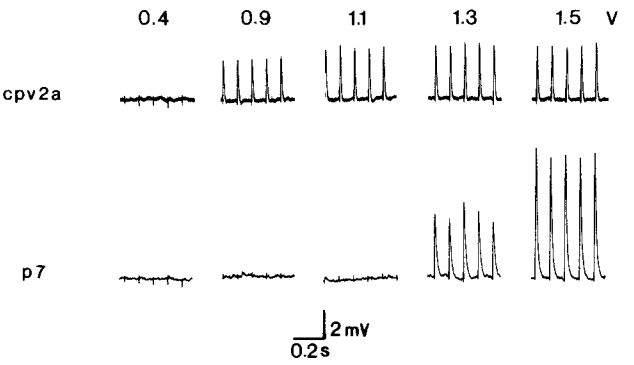


Fig. 5. Separate activation of the PD neurons that innervate intrinsic and extrinsic muscles. Simultaneous intracellular recordings from muscles cpv2a and p7. A suction electrode was used to stimulate lvn, and stimulating voltage was varied. At low voltages no EJPs were evoked. As stimulating voltage was increased, first two discrete thresholds were found that evoked EJPs in muscle cpv2a, then two discrete thresholds were found that evoked EJPs in muscle p7

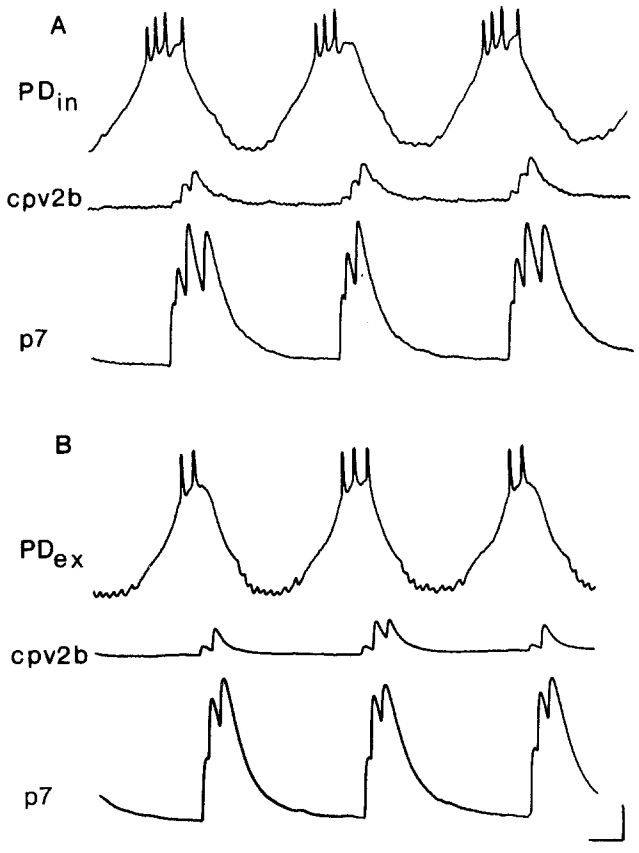
PD neurons. In *P. interruptus* there are two electrically coupled PD neurons that innervate exclusively the extrinsic pyloric dilator and dorsal dilator muscles, cpv1a, b and cpv2b. However, in *C. borealis*, simultaneous intracellular recordings from muscle cpv2b, and the intrinsic muscles p7 and p10, show that p7 and p10 also depolarize simultaneously with cpv2b (Fig. 4). Similar experiments in the crab revealed that the intrinsic muscles p3 and p15 depolarize simultaneously with cpv2b as well. This was surprising, since in *P. interruptus* these *p* muscles are innervated by PY neurons (Maynard and Dando 1974; Govind et al. 1975; Hartline et al. 1986). In *C. borealis* we call muscles p3, p7, p10 and p15 intrinsic PD-phase muscles since they are active in the PD neuron phase of the pyloric rhythm. Several lines of evidence that follow suggest that two of the four PD neurons in the crab innervate the extrinsic cpv1a, b and cpv2a, b muscles, and that the other two PD neurons innervate the intrinsic PD-phase muscles.

First, methylene blue stained preparations of the cpv1a, b and cpv2a, b muscles in *C. borealis* showed two large axons branching over the surface of these muscles, suggesting that these muscles are innervated by two motorneurons. Methylene blue stained preparations of the intrinsic PD-phase muscles also showed two large axons. Second, recordings from the pdn showed two discrete units that fired 2–5 ms apart in doublets. The rise-time of the EJPs is 10–30 ms, longer than the time between the doublets in pdn recordings. Therefore each unitary EJP probably is due to the summated action of two PD motorneurons.

Third, when the lvn, containing the axons of the four PD neurons, was stimulated with a suction electrode, four voltage thresholds that induced EJPs in PD phase muscles were found. Figure 5 shows recordings of an experiment in which the voltage applied to the lvn stimulating electrode was varied in small steps. At 0.4 V applied to the stimulating electrode there were no EJPs evoked. At 0.9 V a single EJP was evoked in muscle cpv2a. At 1.1 V another EJP was evoked in cpv2a; still no EJPs were evoked in muscle p7. At higher voltages two discrete EJPs were evoked in muscle p7 but no increment was seen in the amplitude of the EJPs in muscle cpv2a (Figure 5). In other experiments we were able to show that all the intrinsic PD phase muscles (p3, p7, p10, and p15) shared innervation (EJPs were evoked at the same threshold in all muscles) and the extrinsic PD phase muscles shared innervation (cpv1a, b and cpv2a, b). In no case was any threshold voltage found that induced simultaneous EJPs in both an intrinsic and an extrinsic PD phase muscle.

Fourth, although the intrinsic and extrinsic PD phase muscles were depolarized simultaneously in the pyloric rhythm, they showed different patterns of EJPs. For example, in Fig. 4 the two intrinsic PD phase muscles (p7 and p10) received four EJPs during each depolarization while the extrinsic PD phase muscle received five, suggesting that different neurons innervate the intrinsic and extrinsic muscles.

The intracellular identification of the two classes of PD neurons is shown in Figs. 6 and 7. Figure 6A shows intracellular recordings from a PD



Fi. 6A, B. Identification of PD neurons. **A** Simultaneous intracellular recordings from a PD_{in} neuron and muscles cpv2b and p7. **B** Same preparation as A, simultaneous intracellular recordings from a PD_{ex} neuron and cpv2b and p7. Trough of the PD_{in} neuron, −70 mV; the PD_{ex} neuron, −42 mV. Resting potentials: cpv2b, −66 mV; p7, −59 mV. Vertical calibration, 4 mV for PD neurons, 2 mV for muscle recordings; horizontal calibration 100 ms

neuron and from the cpv2b and p7 muscles. Action potentials in this PD neuron are followed one for one by EJPs in the p7 muscle, but not in muscle cpv2b. Figure 6B shows the same muscles, from the same experiment, but this time with an intracellular recording from another PD neuron. Here the action potentials in each burst of this PD neuron matched the EJPs in the cpv2b muscle, but not in p7. When the PD neuron shown in Fig. 6B was hyperpolarized (Fig. 7), the EJPs disappeared from muscle cpv2b, but the activity in muscle p7 was unaffected. These data again argue that different neurons innervate the intrinsic p7 and extrinsic cpv2b muscles.

We have operationally named the PD neurons that innervate the intrinsic PD phase muscles PD_{in} (PD-intrinsic) neurons and the PD neurons that innervate the extrinsic PD phase muscles PD_{ex} (PD-extrinsic) neurons. Our data suggest that all the PD innervated muscles are each innervated by only two motorneurons, and therefore it appears that there are two each of the two classes of neurons. Preliminary experiments show that some degree of electrical coupling exists between all the PD neurons. Since the extrinsic and intrinsic muscles usually receive different patterns of EJPs, it is likely that the two PD neurons of a single class are tightly electrically coupled and fire in almost synchronous doublets, resulting in a single, summated EJP, and also likely that the two classes of neurons are less tightly electrically coupled. This would explain why hyperpolarization of one PD_{ex} neuron in Fig. 7 could both suppress all EJPs in extrinsic muscles but have no obvious effect on EJPs recorded in intrinsic muscles.

We were interested to determine whether any of the known neural inputs to the STG appeared to have different effects on these two classes of PD neurons. In *P. interruptus* the inferior ventricular (IV) neurons in the brain are a well-defined input to the pyloric system (Claiborne and Selverston 1984). Stimulation of the inferior ventricular nerve (IVN) results in extensive modulation of the pyloric motor pattern as a result of synaptic connections that the IV neurons make with pyloric motorneurons (Claiborne and Selverston 1984; Selverston et al. 1976; Sigvardt and Mulloney 1982; Marder and Eisen 1984b).

The results of IVN stimulation in *C. borealis* are shown in Fig. 8. Stimulation of the IVN produced an increase in the frequency and intensity of the pyloric rhythm. Additionally, subsequent to IVN stimulation 'trailing' EJPs (arrows) appeared in the recording from muscle p7 (innervated by PD_{in}) but not in the cpv1b and cpv2b muscles (innervated by the PD_{ex} neurons). Intracellular recordings from the somata of PD_{in} and PD_{ex} neurons also show different patterns of activity subsequent to IVN stimulation (M. Nusbaum and V. Budnik, unpublished results). These data suggest that the two classes of PD neurons are differently modulated by neuronal inputs, as was originally proposed by Dando et al. (1974).

PY neurons. In *Panulirus* there are approximately eight PY neurons. These have not been individually identified according to which muscle they innervate, but instead have been identified as PY neurons because they have axons in the pyn, and are inhibited by the PD neurons and the LP neuron (Maynard 1972; Eisen and Marder 1982). The PY neurons in *P. interruptus* have been operationally classified into two groups according to when they fire relative to the LP neuron and whether they

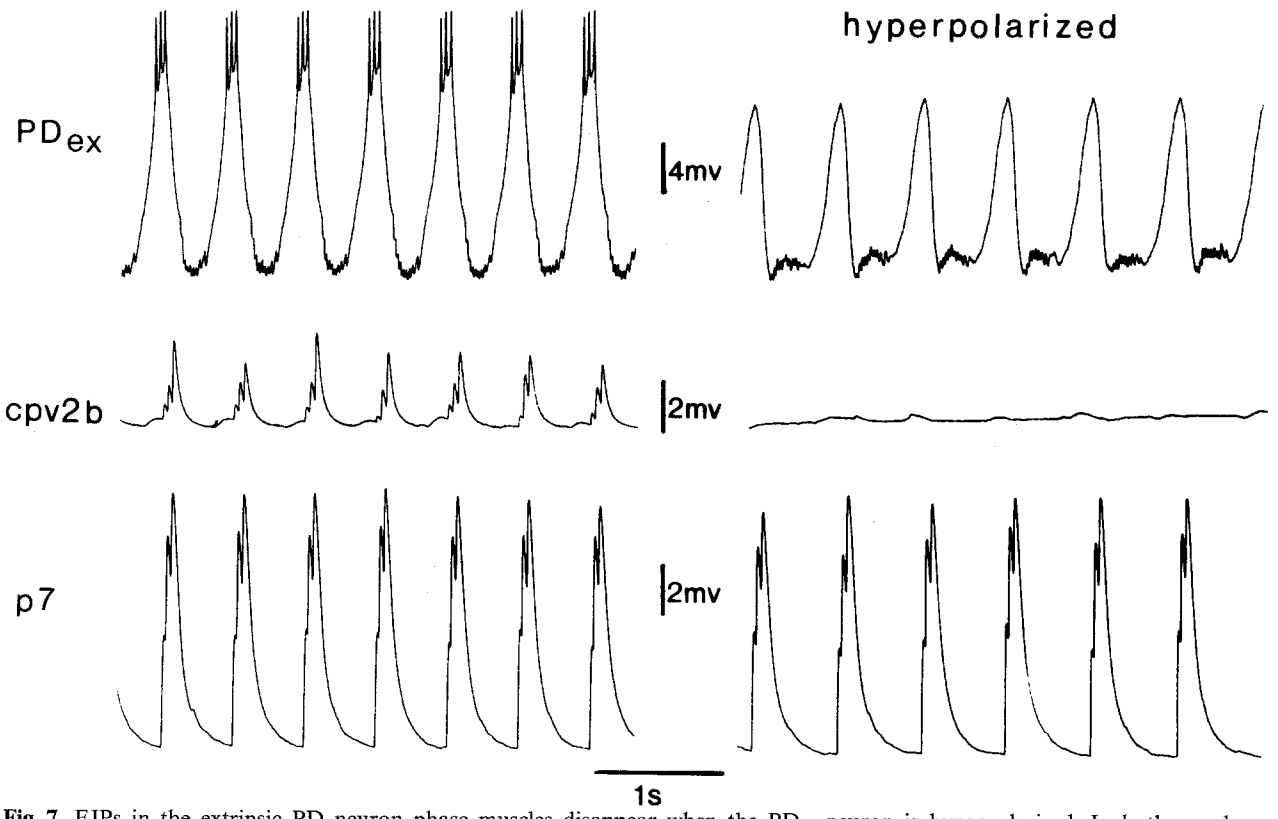


Fig. 7. EJPs in the extrinsic PD neuron phase muscles disappear when the PD_{ex} neuron is hyperpolarized. In both panels are shown simultaneous intracellular recordings from a PD_{ex} neuron and muscles cpv2b and p7. Left: no polarizing current. Right: PD_{ex} neuron hyperpolarized by current injection (−2 nA) through a second electrode, thus preventing the neuron from firing. The trough of the PD_{ex} neuron membrane potential was −70 mV in left panel and −86 mV in right panel. Resting potentials of muscle fibers were −66 mV for cpv2b and −59 mV for p7. The residual small oscillations seen in the right hand recording for cpv2b are movement artifacts due to contraction of the other pyloric region muscles

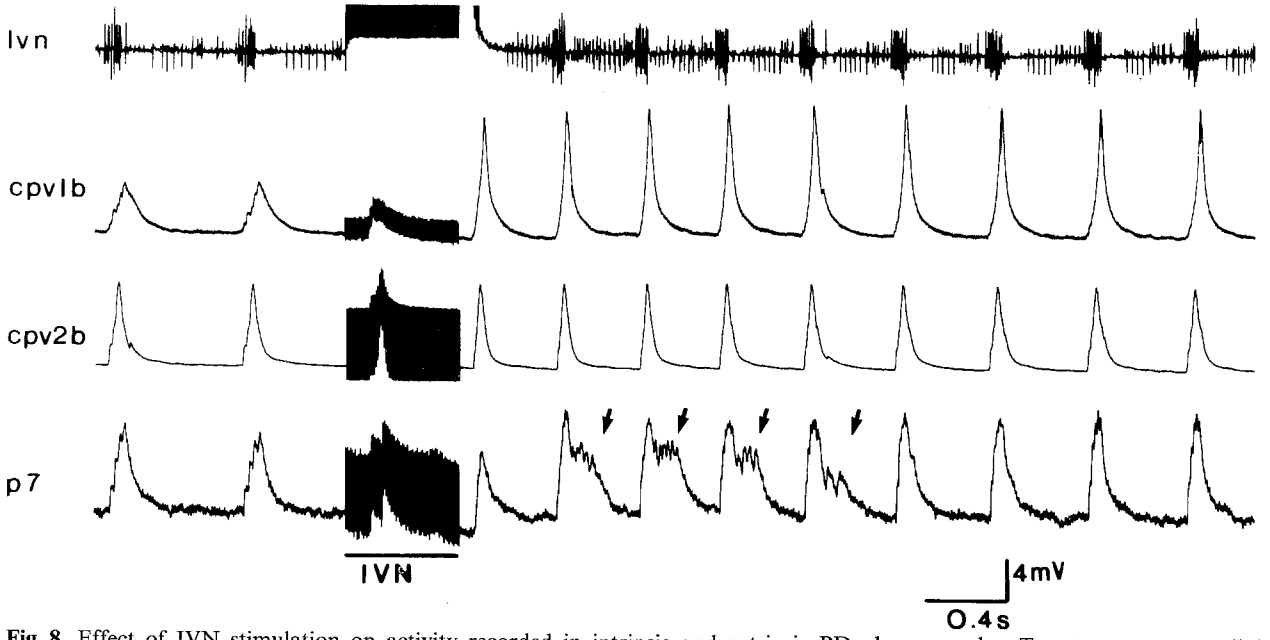


Fig. 8. Effect of IVN stimulation on activity recorded in intrinsic and extrinsic PD phase muscles. Top trace an extracellular recording from the lvn. Other three traces are simultaneous intracellular recordings from muscles cpv1b, cpv2b and p7. IVN was stimulated at the bar at 60 Hz for 500 ms. Following IVN stimulation trailing EJPs (arrows) are seen in the p7 recording that are not visible in the recordings from cpv1b and cpv2b. Resting potentials: cpv1b, −72 mV; cpv2b, −70 mV; p7, −68 mV

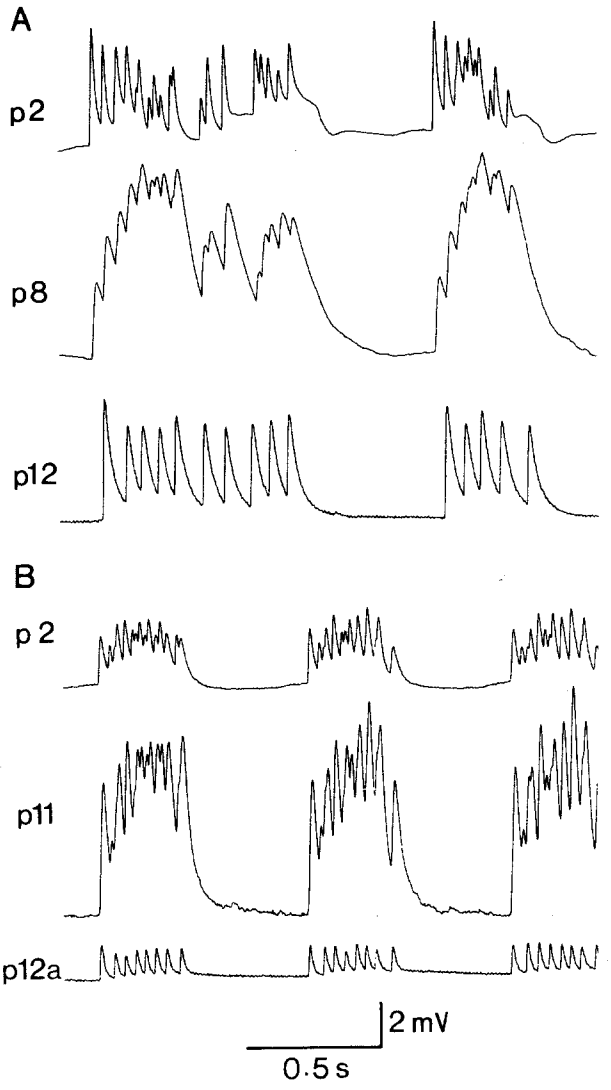


Fig. 9A, B. PY neuron innervated muscles during rhythmic pyloric activity. **A** simultaneous intracellular recordings from muscles p2, p8, and p12. **B** simultaneous intracellular recordings from muscles p2, p11, and p12a. Resting potentials: p2, −58 mV; p8, −36 mV; p11, −35 mV; p12, −48 mV; p12a, −30 mV

receive a synaptic input from the hepatopancreas duct nerve (Hartline et al. 1979; Hartline and Gassie 1979; Hartline et al. 1986). The innervation of this region of the pylorus has been partially characterized in *P. interruptus* (Govind et al. 1975; Hartline et al. 1986). However, it is still not completely clear whether the PY neuron innervated muscles are divided into two or more classes that contract at different times in *P. interruptus*. To determine if the PY neurons are individually identifiable in *C. borealis*, we have systematically attempted to determine how many neurons innervate each of these muscles, and whether these muscles are active at different times.

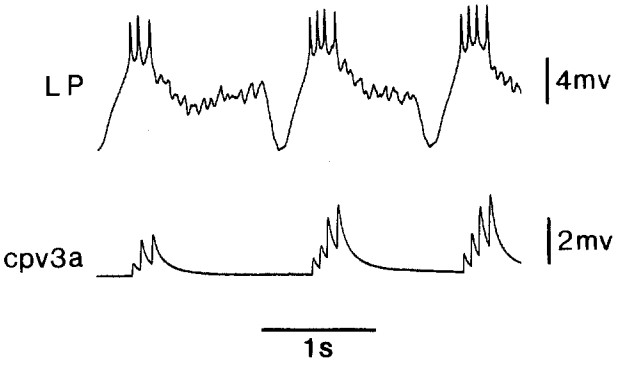


Fig. 10. LP neuron innervates muscle cpv3a. Top trace is an intracellular recording from a LP neuron, bottom trace from cpv3a. Trough of the LP neuron membrane potential was −60 mV. Resting potential, cpv3a, −59 mV

In *C. borealis* all the muscles innervated by the PY neurons are active at the same time in the pyloric cycle (Fig. 9). Attempts to divide the PY neurons into subgroups by stimulation of the lvn while recording intracellularly from these muscles have been partially successful. There appear to be at least two sets of PY neurons, two neurons that innervate p2 and p8 and two that innervate p11. We are unable to say whether some combination of these four neurons, or still another set of neurons, innervate muscles cpv11, p12–14, and p12a. Methylene blue staining has been unable to resolve this issue due to the complicated anatomy and branching patterns of the nerves in this region.

LP neuron. In *P. interruptus* the single lateral pyloric (LP) neuron innervates muscles cpv4, cpv5, p1, and probably cpv7 (Maynard and Dando 1974). Figure 10 shows intracellular recordings from an LP neuron and muscle cpv3a , and shows that each action potential in the LP neuron soma is associated with a discrete EJP, just as Fig. 3 showed for muscle p1. Similar recordings revealed that muscles p1, cpv3a, cpv3b, cpv4, cpv6, and cpv8 are all innervated by the LP neuron. When the nerve innervating these muscles was stimulated, a single threshold voltage was found to induce simultaneous EJPs in all these muscles. Furthermore, methylene blue staining revealed a single large axon over the surface of these muscles.

VD and IC neurons. In *P. interruptus* there is a single ventricular dilator (VD) neuron that innervates muscle cv1 and a single inferior cardiac (IC) neuron that innervates muscle cv2 and possibly muscle cv3 (Maynard and Dando 1974). The axons of these neurons travel in the medial ventricular nerve (mvn). The branching pattern of the mvn

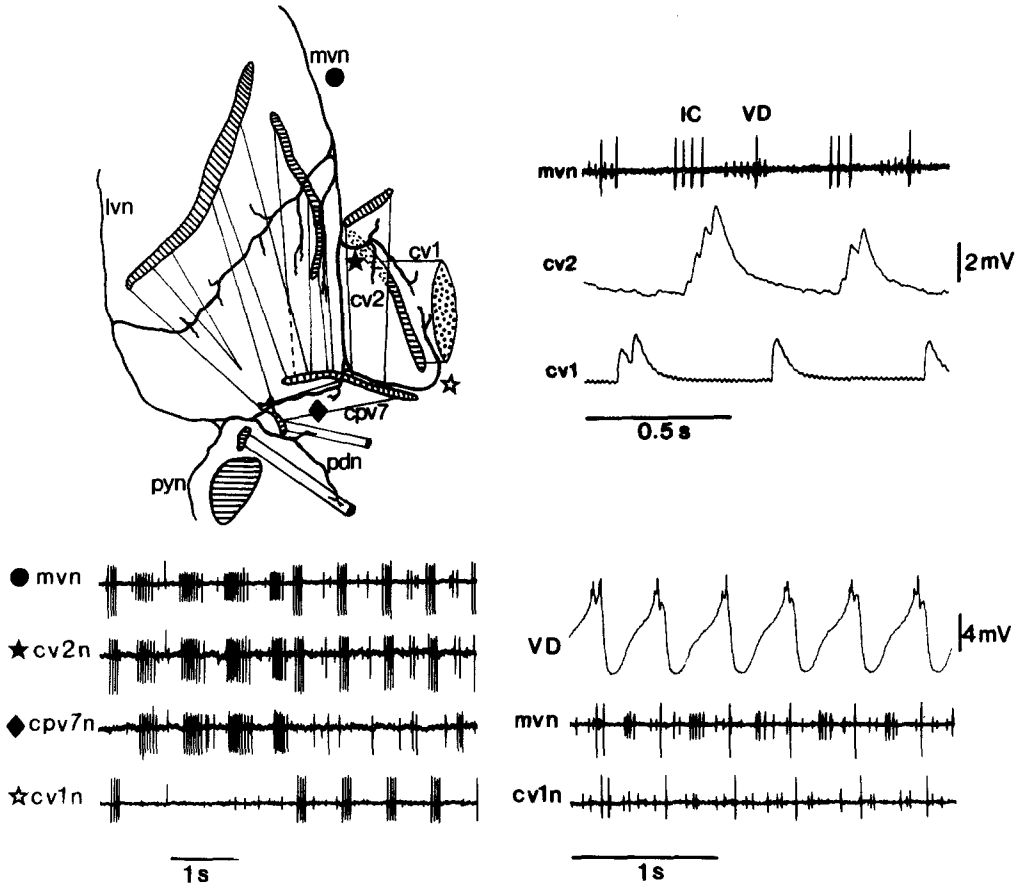


Fig. 11. Branches of the mvn and the innervation patterns of the VD and IC neurons. Drawing shows branching pattern of the mvn. Symbols on the drawing indicate position of the extracellular recording shown below. The top right shows simultaneous recordings from the mvn (extracellular), and muscles cv2 and cv1. Action potentials due to the activity of the IC neuron are followed by one-to-one constant latency EJPS in muscle cv2. VD neuron action potentials evoke EJPs in muscle cv1. Resting potentials: cv2, −42 mV; cv1, −70 mV. Bottom right panel illustrates identification of the VD neuron. Intracellularly recorded action potentials correlate 1 for 1 with extracellularly recorded action potentials on the cv1 nerve (cv1n)

in *C. borealis* is shown in the diagram of Fig. 11. Simultaneous intracellular recordings from the cv1 and cv2 muscles show that they are innervated by different neurons (as in *P. interruptus*) with axons in the mvn. Since the VD and IC action potentials in the mvn are often similar in amplitude, in order to identify the VD and IC neurons it is useful to place recording electrodes on branches innervating single muscles, as shown in Fig. 11. The extracellular recordings in the bottom left hand panel show the activity patterns recorded at the branches of the mvn indicated. The mvn and cv2n recordings show activity of both the VD and IC neurons. This figure shows that electrodes placed on the cv1n and the cpv7n allow the separate recording of the VD and IC neurons, respectively. Using a cv1n recording the VD neuron can be uniquely identified in intracellular recordings (bottom right panel).

Stimulation of the mvn revealed a single threshold voltage that induced EJPs in muscle cv1, and a single different threshold voltage that induced simultaneous EJPs in muscles cv2 and cpv7. Methylene blue staining revealed two to three large axons in the mvn, but only a single large axon in the branch of the mvn innervating the cv1 muscle, and another single large axon in that innervating the cv2 and cpv7 muscles. Although almost all mvn recordings show a small unit in addition to the VD and IC, this unit does not appear to innervate the cv1, cv2 or cpv7 muscles, but its activity appears correlated with the gastric mill rhythm.

Neurotransmitters used by the pyloric motor neurons

In *P. interruptus* the PD and VD motorneurons make cholinergic neuromuscular junctions

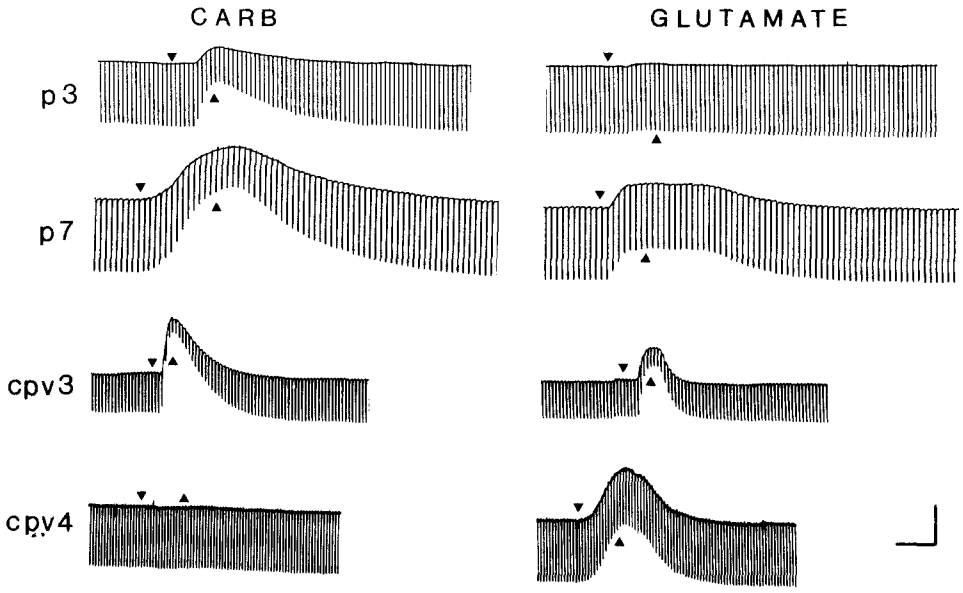


Fig. 12. Sensitivity of pyloric region muscles to Carb and glutamate. Saline contained 20 m*M* $MnCl_2$ to prevent muscle contractions and to block all presynaptic transmitter release. In all experiments two electrodes were placed in each muscle fiber, one to pass current pulses of constant amplitude while the other monitored membrane potential. In all cases only voltage recording is shown, but the current was monitored and did not change during the recordings shown. Downward arrows denote the time at which the manifold was switched to the port leading to the Carb (5×10^{-4} *M*) or glutamate (5×10^{-4} *M*) containing saline, the upward arrows indicate when the wash was started. Vertical calibration: 10 mV p3, p7; 20 mV cpv3, cpv4. Horizontal calibration: 20 s. Resting potentials: p3, −62 mV; p7, −70 mV; cpv3, −72 mV; cpv4, −54 mV. Current pulses: p3, −640 nA; p7, −160 nA; cpv3, −14 nA; cpv4, −220 nA

(Marder 1974, 1976) while the LP, PY, and IC neurons make non-cholinergic, and almost certainly glutamatergic neuromuscular junctions (Marder 1976; Lingle 1980). The PD_{in} neurons are active in PD phase and are electrically coupled to the PD_{ex} neurons (that are homologous to the PD neurons in *P. interruptus*) but innervate muscles that receive glutamatergic innervation in *P. interruptus*. Therefore, we investigated the sensitivity of all the pyloric muscles to both ACh and glutamate. Additonally, since curare blocks the EJPs evoked by stimulation of the cholinergic PD and VD neurons in *P. interruptus* (Marder 1974, 1976) by its action on postjunctional cholinergic responses (Marder and Paupardin-Tritsch 1980), we checked the sensitivity of the EJPs evoked by each of the pyloric group neurons to blockade by curare.

Figure 12 shows the responses of muscles p3 and p7 (PD_{in}-innervated) and cpv3 and cpv4 (LP neuron innervated) to bath applied carbamylcholine (Carb), a non-hydrolyzable cholinergic agonist, and to glutamate. Muscles p3 and p7 depolarized in response to a short pulse of 5×10^{-4} *M* Carb. This depolarization was associated with an increase in conductance of the muscle membrane. Muscle p7 but not p3 also depolarized in response to a short pulse of 5×10^{-4} *M* glutamate. Both the LP neuron-innervated muscles depolarized in response to glutamate, but only one of them, muscle cpv3 also depolarized in response to Carb. Iontophoretic applications of ACh and glutamate confirmed the results of bath application of agonists.

The results of similar experiments on representative muscles innervated by each of the pyloric motorneurons are summarized in Table 1. All the muscles innervated by motorneurons that are cholinergic in *P. interruptus* (the PD and VD neurons) were sensitive to cholinergic agonists. All those innervated by motorneurons that are glutamatergic in *P. interruptus* (the PY, LP, and IC neurons) were sensitive to glutamate. Additionally, several of these muscles showed robust depolarizing, conductance increase responses to both Carb and glutamate, as has also been seen in other species (Lingle 1980).

Figure 13 shows the effects of curare on the amplitude of EJPs recorded in various pyloric muscles during ongoing pyloric activity. Figure 13A shows that the application of 10^{-4} *M* curare resulted in a reduction of 50–70% in the amplitude of the EJPs in muscles cpv2b (PD_{ex}-innervated) and p7 (PD_{in}-innervated). Recordings from the lvn (not shown) revealed that the curare application had no effect on the ongoing pyloric rhythm. In Figure 13B, EJPs were evoked by stimulation of

Table 1. Innervation of the pyloric region

Muscle name	*C. borealis* motor neuron	*P. interruptus* motor neuron	EJPS Curare	Carb sensitive	Glutamate sensitive
cpv1a, b	PD_{ex}	PD	+	+	−
cpv2a, b	PD_{ex}	PD	+	+	−
p3	PD_{in}	PY	+	+	−
p7	PD_{in}	PY	+	+	+
p10	PD_{in}	PY	+	+	+
p15	PD_{in}	not present			
p2	PY	PY	−	+	+
p8	PY	PY	−	−	+
p11	PY	PY	−	−	+
p12–14	PY	PY	−		
cpv11	PY	not present	−		
p1	LP	LP	−	−	+
cpv3a, b	LP	LP		+	+
cpv4	LP	LP		−	+
cpv8	LP	not present		−	+
cpv6	LP	not present	−	−	+
cv2	IC	IC	−	−	+
cpv7	IC	LP			+
cv1	VD	VD	+	+	+

+ = yes
− = no
not present refers to muscles not found in *P. interruptus* but present in crabs

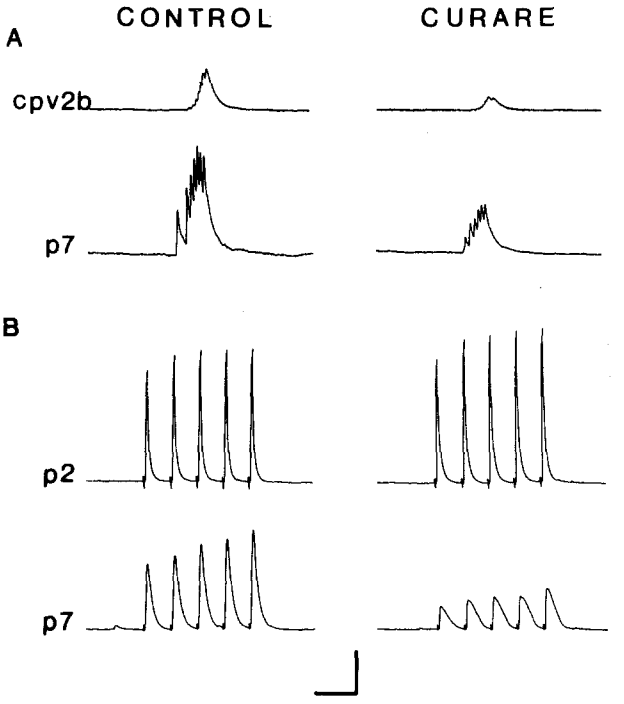


Fig. 13A, B. Effect of curare on EJPs evoked by PD and PY neurons. **A** EJPs recorded intracellularly in muscles cpv2b and p7 during rhythmic pyloric activity. Amplitudes of EJPs recorded in both muscles were reduced 50–70% by application of 10^{-4} *M* curare. The effect of curare was reversed after long washes (not shown). Curare had little or no direct effect on muscle fiber input impedance (not shown). Vertical calibration: cpv2b, 4 mV; p7, 2 mV;. Horizontal calibration: 0.5 s. Resting potentials: cpv2b, −60 mV; p7, −56 mV. **B** EJPs evoked by stimulation of the nerve. Amplitude of EJPs in p7 was reduced 60% by 10^{-4} *M* curare but the EJPs in p2 (PY neuron innervated) were unaffected. Vertical calibration: 4 mV. Horizontal calibration: 0.3 s. Resting potentials: p2, −70 mV; p7, −64 mV

the lvn. In this experiment 10^{-4} *M* curare had no effect on the PY neuron-evoked EJPS in muscle p2, but produced a 60% decrease in the amplitude of the EJPs in muscle p7 (PD_{in}-innervated).

The results of similar experiments performed on representative muscles innervated by each of the pyloric motorneurons are summarized in Table 1. The amplitudes of the EJPs in all muscles innervated by the putatively cholinergic motorneurons (PD and VD neurons) were decreased by curare, while those of muscles innervated by putatively glutamatergic motorneurons (PY, LP, and IC neurons) were not. All muscles whose EJPs were reduced in amplitude by curare were sensitive to Carb, but only some were also sensitive to glutamate. All muscles whose EJPs were curare resistant were sensitive to glutamate, but only some were Carb sensitive. These data, taken together, support the contention that the PD_{in}, PD_{ex}, and VD neurons are cholinergic and the PY, LP, and IC neurons are glutamatergic.

Discussion

Very little is known concerning the evolution of neural circuits. Homologous neurons and muscles have been identified and compared in many arthropod preparations (Kahan 1971; Meiss and Norman 1977a, b; Paul 1985a, b; Paul et al. 1985; Pearson et al. 1985; Schaefer 1970; Wiens and Rathmayer 1985; Wilson and Hoyle 1978; Govind and Wiens 1985; Wiens and Govind 1985). In none of these systems, however, is it yet possible to simultaneously compare the neuronal outputs and synaptic connectivities of homologous neural circuits. The pyloric system of the crustacean stomatogastric ganglion provides a preparation in which a comparison of the motor outputs and neural circuits in several decapod species is possible. This will provide a better understanding both of how the pyloric neuromuscular system has changed during evolution and of the constraints on the natural design of neural circuits.

Throughout this paper we refer to anatomically similar muscles in different species as homologous, as did previous authors (Maynard and Dando

1974; Meiss and Norman 1977a, b; Schaefer 1970). We believe that the stomatogastric system muscles are evolutionarily homologous as the term is defined by Ghiselin (1966) in that muscles in different species can be traced back to a single muscle present in a common ancestor of crabs and palinuroid lobsters. To the extent to which the phylogenetic relationships of crustaceans are understood, a systematic comparative study of the stomatogastric musculature in crustaceans would lead to unambiguous definitions of homologous muscles. However, the phylogenetic relationships of the decapod crustacean species are still under study (Schram 1982). It is interesting that in the Penaedean shrimp, *Palaemon serrahes*, the stomach is sufficiently similar to that of lobsters and crabs to identify the major muscle groups (Meyrand and Moulins 1986). This suggests that it may be possible to use the innervation patterns and anatomy of the stomachs of present day decapod crustaceans as an aid in determining the phylogenetic relationships among crustaceans. In spite of possible theoretical difficulties in defining homology, in practice the arrangements of muscles, ossicles, and nerves in these decapods are extremely constant from species to species, and recognizing a given muscle in several species is quite easy (Maynard and Dando 1974; Govind et al. 1975; Lingle 1980). In fact, there appear to be more differences in the innervation patterns from species to species than in the anatomical forms of muscles and ossicles.

It is interesting that homologous muscles in *P. interruptus* and *C. borealis* are thought to receive a glutamatergic innervation in *P. interruptus* (Lingle 1980) but are innervated by motorneurons likely to be cholinergic in *C. borealis* (Table 1). For example, muscle p10 is innervated by a glutamatergic PY neuron in *P. interruptus* (Govind et al. 1975; Hartline et al. 1986; Lingle 1980) but a cholinergic PD_{in} neuron in *C. borealis*. Many of the stomatogastric muscles display both glutamatergic and cholinergic receptors even though they are thought to receive innervation by motorneurons releasing only one or the other neurotransmitter (Lingle 1980; Fig. 12). These extra receptors might be vestiges of innervation from a neuron using the other neurotransmitter that was present either at an earlier evolutionary or developmental stage.

The present work is the initial step in obtaining a full description of the pyloric system in *C. borealis*. The pyloric motor patterns of the crab are very similar to those of *P. interruptus*, the preparation that has been studied in most detail. However, it has been known for a long time that there are four neurons that fire during the PD phase of the pyloric rhythm in crabs (Dando et al. 1974; Hermann and Dando 1977; Hermann 1979a, b) rather than the 2 PD neurons found in lobsters (Maynard 1972). This paper clearly shows that these four PD neurons are not identical, as was suggested by earlier work (Dando et al. 1974). Rather, there are two PD_{ex} neurons that ressemble closely the PD neurons in lobsters. Additionally, there are two other neurons, the PD_{in} that are clearly different from any class of neurons in lobsters, but have some features in common with PD, PY, and LPG neurons in *P. interruptus*.

Although we have not yet determined the pattern of synaptic connectivity among the neurons of the *C. borealis* STG, all four of the crab PD neurons seem similar in this regard to the two PD neurons of *P. interruptus*. For example, LP neuron evoked inhibitory postsynaptic potentials (IPSPs) can be recorded in all the PD neurons, and all four appear to make cholinergic neuromuscular junctions.

Our data are consistent with there being five or six PY neurons in *C. borealis* rather than the eight PY neurons reported in *P. interrruptus* (Maynard and Dando 1974; Selverston et al. 1976). Since the PD_{in} neurons innervate muscles thought to be innervated by the PY neurons in *P. interruptus*, it is possible that two neurons that during development become PY neurons in *P. interruptus* become PD_{in} neurons in *C. borealis*. The VD, IC, and LP neurons in *C. borealis* and *P. interruptus* innervate homologous muscles, with the exception that muscle cpv7 is innervated by the IC neuron in *C. borealis* but the LP neuron in *P. interruptus*.

What behavioral significance do the differences in the innervation patterns and synaptic connections shown in these different decapod species have? The pyloric region of the stomach is believed to function as a filter. It is clear that some of the muscles in the pyloric region of *C. borealis* contract at different times in the motor pattern than do their homologues in *P. interruptus*. The exact pattern of motion of this region may not be particularly important to its function, and therefore this change may have no significance. This seems unlikely since this system is subject to extensive modulation with respect to the frequency, phase relationships, and burst lengths of its neurons both *in vivo* (Rezer and Moulins 1983) and in isolated preparations (Beltz et al. 1984; Eisen and Marder 1984; Hooper and Marder 1984; Marder and Hooper 1985; Harris-Warrick 1986).

A second possibility is that the different pat-

terns of movement in the stomachs of decapods reflect specializations in stomach function that confer selective advantages to each species as a result of differences in diet or metabolic requirements, as suggested by Schaefer (1970). A third possibility is that the changes in innervation pattern seen in crabs and lobsters may preserve identical stomach motion and function by offsetting effects of anatomical differences in the stomachs of these animals that result from the differences in body form found in crabs and lobsters. In any case, it is clear that in different species nearly identical motor patterns driving areas of very similar anatomy, can still result in quite different movements.

Comparative studies of the stomatogastric system may yield insight into how neuromuscular systems change and evolve the ability to produce more complex and varied movements. One possible way for a neuromuscular system to evolve the capacity for finer control of its motor output and provide for a wider range of motor outputs, is to simultaneously increase the number of distinct classes of motor neurons while decreasing the number of muscles (or muscle fibers in the case of vertebrates) that each individual neuron innervates.

In *P. interruptus* functionally distinct neurons that are electrically coupled and fire simultaneously respond differently to the same synaptic and modulatory inputs (Marder and Eisen 1984b). In *C. borealis* the PD_{in} and PD_{ex} neurons also respond differently to the activation of a synaptic input, that from the IVN fibers. After IVN stimulation the muscles innervated by the PD_{in} neurons remain depolarized after those innervated by the PD_{ex} neurons are no longer depolarized. The ability to differentially modulate the activity of the PD_{ex}, PD_{in} and PY neurons may increase the number of degrees of freedom of the pyloric region. Thus it is possible that the crab stomach may be capable of a greater repertoire of distinct movements than stomachs of animals with only 2 PD neurons.

As the details of the synaptic connectivity among the neurons of the stomatogastric ganglion in a large number of decapod species become available, it should be possible to use these preparations to provide insights into the rules governing the evolution of neural circuits.

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